Rapid Communication

C-Peptide stimulates Na^+, K^+ -ATPase activity via PKC α in rat medullary thick ascending limb

M. Tsimaratos¹, F. Roger², D. Chabardès³, D. Mordasini², U. Hasler², A. Doucet³, P.-Y. Martin², E. Féraillé²

¹ Paediatric Nephrology Unit, Children's Hospital la Timone, Marseille, France

² Nephrology Laboratory, Foundation for Medical Research, Geneva, Switzerland

³ Cell Biology Unit, CNRS URA 1859, Gif-Sur-Yvette, France

Abstract

Aims/hypothesis. C-peptide, the cleavage product of proinsulin processing exerts physiological effects including stimulation of Na^+, K^+ -ATPase in erythrocytes and renal proximal tubules. This study was undertaken to assess the physiological effects of connecting peptide on Na^+, K^+ -ATPase activity in the medullary thick ascending limb of Henle's loop.

Methods. Na^+, K^+ -ATPase activity was measured as the ouabain-sensitive generation of ^{32}Pi from $\gamma[^{32}\text{P}]\text{-ATP}$ and ^{86}Rb uptake on isolated rat medullary thick ascending limbs. The cell-surface expression of Na^+, K^+ -ATPase was evaluated by Western blotting of biotinylated proteins, and its phosphorylation amount was measured by autoradiography. The membrane-associated fraction of protein kinase C isoforms was evaluated by Western blotting.

Results. Rat connecting peptide concentration-dependently stimulated Na^+, K^+ -ATPase activity with a threshold at 10^{-9} mol/l and a maximal effect at 10^{-7} mol/l. C-peptide (10^{-7} mol/l) already stimulates

Na^+, K^+ -ATPase activity after 5 min with a plateau from 15 to 60 min. C-peptide (10^{-7} mol/l) stimulated Na^+, K^+ -ATPase activity and ^{86}Rb uptake to the same extent, but did not alter Na^+, K^+ -ATPase cell surface expression. The stimulation of Na^+, K^+ -ATPase activity was associated with an increase in Na^+, K^+ -ATPase α -subunit phosphorylation and both effects were abolished by a specific protein kinase C inhibitor. Furthermore, connecting peptide induced selective membrane translocation of PKC- α .

Conclusion/interpretation. This study provides evidence that in rat medullary thick ascending limb, C-peptide stimulates Na^+, K^+ -ATPase activity within a physiological concentration range. This effect is due to an increase in Na^+, K^+ -ATPase turnover rate that is most likely mediated by protein kinase C- α phosphorylation of the Na^+, K^+ -ATPase α -subunit, suggesting that C-peptide could control Na^+ reabsorption during non-fasting periods. [Diabetologia (2003) 46:124–131]

Keywords C-peptide, Na^+, K^+ -ATPase, protein kinase C, medullary thick ascending limb, sodium.

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Corresponding author: Dr. M. Tsimaratos, Unité de Néphrologie Pédiatrique, Hôpital d'Enfants de la Timone, 13385 Marseille Cedex 05, France e-mail: michel.tsimaratos@ap-hm.fr

Abbreviations: AQP2, aquaporin-2; AVP, arginine-vasopressin; BCA, bisinchoninic acid; cAMP, cyclic AMP; C-peptide, connecting peptide; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; MTAL, medullary thick ascending limb of Henle's loop; NP-40, nonidet P-40; PAGE, poly-acrylamide gel electrophoresis; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol myristic acid; PVDF, polyvinyl difluoride.

The connecting peptide (C-peptide), a cleavage product of proinsulin processing, and insulin are secreted in equimolar amounts in the bloodstream. A major role of C-peptide is to enable proper folding of the proinsulin molecule by facilitating the formation of disulfide bonds within A-chains and B-chains [1, 2]. The physiological role of secreted C-peptide is still highly debated. Several investigators have shown that C-peptide does not alter blood glucose concentrations but exerts other biological effects through specific binding to a yet unidentified receptor [3]. In healthy human volunteers, C-peptide infusion has no measurable effects since receptors are saturated at ambient

C-peptide concentration [4]. Conversely, in experimental Type 1 diabetes, a situation with complete insulin and C-peptide deprivation, short term infusion of C-peptide increases renal functional reserve, reduces protein leakage and improves hyperfiltration [5]. At the cellular level, the effects of C-peptide include stimulation of Na⁺,K⁺-ATPase activity [6, 7] and activation of endothelial nitric oxide synthase [8, 9, 10]. The C-peptide signal transduction pathway is thought to involve an heteromultimeric G-protein-mediated increase in intracellular Ca²⁺ leading to protein kinase C (PKC) and mitogen-activated protein kinase activation [4, 6, 11].

Na⁺,K⁺-ATPase is an ubiquitous membrane-bound enzyme composed of at least two subunits: the large 10 transmembrane spanning α -subunit carries the catalytic and ion transport properties while the smaller, single-membrane-spanning β -subunit is involved in Na⁺,K⁺-ATPase maturation and modulates its transport activity. The basic function of the Na⁺,K⁺-ATPase is to maintain high Na⁺ and K⁺ gradients across the plasma membrane of animal cells. In renal tubule epithelial cells, Na⁺,K⁺-ATPase provides the driving force for active Na⁺ reabsorption [12] and its activity under multihormonal control [13].

In the thick ascending limb of Henle's loop active Na⁺,K⁺-ATPase-dependent Na⁺ reabsorption is essential for Na⁺ balance since this nephron segment reabsorbs close to 15% of the Na⁺ filtered load. In addition active Na⁺ reabsorption in the medullary thick ascending limb (MTAL) allows the generation of the osmotic gradient that ultimately allows urine concentration. Na⁺,K⁺-ATPase activity in MTAL results mostly from a continuous stimulation through the cAMP-PKA-dependent pathway and regulatory inhibition [13]. In rat MTAL, insulin displays a small stimulatory effect on Na⁺ reabsorption [14] probably due to poor expression of insulin receptors [15]. In addition, insulin does not stimulate Na⁺,K⁺-ATPase activity in the thick ascending limb [16]. These observations lead us to hypothesise that C-peptide might be a more important physiological regulator of Na⁺ transport than insulin in this nephron segment. Since the mechanisms of control of Na⁺,K⁺-ATPase by hormones show a high degree of cell specificity [13], this study was therefore designed to show a specific physiological effect of C-peptide on Na⁺,K⁺-ATPase activity in rat MTAL, and to analyse the cellular mechanism of this effect.

Materials and methods

Materials. Studies were carried out on male Wistar rats (body weight 150–200 g) with unrestricted access to water and standard laboratory rat chow. As described previously [17], MTAL-enriched tubular suspensions, referred to as outer medullary tubule suspensions, or microdissected MTAL, were obtained by mechanical dissociation or by microdissection from collagenase-treated kidneys, respectively. All experiments were carried

out in an incubation solution containing: 120 mmol/l NaCl, 5 mmol/l RbCl, 4 mmol/l NaHCO₃, 1 mmol/l CaCl₂, 1 mmol/l MgSO₄, 0.2 mmol/l NaH₂PO₄, 0.15 mmol/l Na₂HPO₄, 5 mmol/l glucose, 10 mmol/l lactate, 1 mmol/l pyruvate, 4 mmol/l essential and nonessential aminoacids, 0.03 mmol/l vitamins, 20 mmol/l N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), and 0.1% (w/v) bovine serum albumin (BSA), pH 7.45. As previously published [17], incubation solution was gassed with 95% O₂-5% CO₂ prior to isolation of tubules and before starting incubation at 37°C in order to prevent a decrease in intracellular ATP content.

Synthetic rat C-peptide II (amino acid sequence: EVEDPQVAQLELGGPGAGDLQTLALEVARQ, from Ferring AB, Malmö, Sweden) was a kind gift of Dr. J. Wahren (Department of Clinical Physiology, Karolinska Institute, Stockholm, Sweden) and its purity was more than 99% as assessed by HPLC [7]. Rabbit polyclonal anti-Na⁺,K⁺-ATPase α -subunit antibody raised against the rat holoenzyme was previously described [18]. Mouse monoclonal anti-PKC- α antibody was from Transduction Laboratories (Lexington, Ky., USA) and rabbit polyclonal antibodies against PKC δ , PKC ϵ , and PKC ζ were from Life Technologies (Carlsbad, Calif., USA). BCA assay, EZ-link sulfo-NHS-S-S-Biotin, and streptavidin-agarose beads were from Pierce (Rockford, Ill., USA). Protein A-sepharose beads, ⁸⁶RbCl, γ -[³²P]-ATP were from Amersham-Pharmacia Biotech (Piscataway, N.J., USA), α -[³²P]-ATP and [³²P]-orthophosphoric acid were from New England Nuclear (Boston, Mass., USA). GF109203X was from Calbiochem (San Diego, Calif., USA) and all other chemicals were from Sigma-Aldrich (St. Louis, Mo., USA). The study was reviewed and approved by the local animal ethics committee.

Na⁺,K⁺-ATPase activity. The hydrolytic activity of Na⁺,K⁺-ATPase was assessed on pools of 4 to 6 microdissected MTAL segments according to a previously described radiochemical assay based on the measurement of Pi released from γ -[³²P]-ATP under V_{max} conditions [19]. After incubation with or without drugs and/or hormones, tubules were permeabilised by hypotonic shock followed by a freezing-thawing step and ATPase activity was measured. The ATPase assay solution contained (mmol/l): 100 NaCl, 10 KCl, 10 MgCl₂, 1 EDTA, 100 Tris HCl, 10 MgATP, and tracer amounts (185 Bq/ μ l) of γ -[³²P]-ATP for total ATPase activity. For measurement of Na⁺,K⁺-independent ATPase activity, NaCl and KCl were omitted, Tris-HCl was 150 mmol/l, and ouabain 2 mmol/l was added. The pH of both solutions was 7.4. In each experiment, total ATPase activity and Na⁺,K⁺-independent ATPase activity were each examined on four replicates. Na⁺,K⁺-ATPase was taken after subtracting the mean Na⁺,K⁺-independent ATPase activity from the mean total ATPase activity, and was expressed as pmol P_ih⁻¹mm⁻¹±SE.

⁸⁶Rubidium uptake. Rubidium (⁸⁶Rb) uptake was assessed on intact microdissected tubules by measuring the intracellular accumulation of ⁸⁶Rb under conditions of initial rate [20]. Ouabain-sensitive ⁸⁶Rb uptake was calculated as the difference between the mean values measured in samples without and with 2.510⁻³ mol/l ouabain, respectively. ⁸⁶Rb uptake was expressed as pmol Rbmm⁻¹min⁻¹±SE.

Biotinylation assay and Western blotting. The Na⁺,K⁺-ATPase cell surface expression was calculated as previously described [21]. After preincubation in the absence of or with C-peptide, pools of 60 microdissected MTALs were incubated with 1.5 mgml⁻¹ sulfo-NHS-S-S-Biotin for 1 hour at 4°C. After cell lysis in homogenization buffer (20 mmol/l Tris-HCl pH 7.4,

2 mmol/l EDTA, 2 mM EGTA, 25 μgml^{-1} leupeptin, 25 μgml^{-1} aprotinin, 1 mM PMSF, 1 mmol/l AEBSF, 30 mmol/l NaF, 30 mmol/l Na^+ pyrophosphate along with 0.1% (wt/vol) SDS and 1% (vol/vol) Triton X100), biotinylated proteins were precipitated by streptavidin-agarose beads diluted in an antiprotease-containing buffer (50 mmol/l Tris-HCl pH 7.4, 100 mmol/l NaCl, 5 mmol/l EDTA, 20 μgml^{-1} leupeptin, 25 μgml^{-1} aprotinin). The beads were washed twice with rinsing solution A (50 mmol/l Tris-HCl pH 7.4, 5 mmol/l EDTA, 50 mmol/l NaCl), twice with rinsing solution B (25 mmol/l Tris-HCl pH 7.4, 500 mmol/l NaCl) and once with 10 mmol/l Tris HCl pH 7.4. After resuspension in Laemmli's sample buffer, proteins were separated by SDS-PAGE, transferred on polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, Mass., USA) and incubated at 4°C with anti- Na^+, K^+ -ATPase antibody diluted 1/10000 (vol/vol) in tris-buffered saline NP-40 medium (TBS-NP40 : 150 mmol/l NaCl, 50 mmol/l Tris, 0.2% Nonidet P-40, pH 7.4) with 5% dried non-fat milk. After washing in TBS-NP40, membranes were incubated with an anti-rabbit IgG antibody (dilution 1/10000 vol/vol) coupled to horseradish peroxidase (Transduction Laboratories) in TBS-NP40. The antigen-antibody complexes were detected by chemiluminescence with the Super Signal Substrate method (Pierce) according to the manufacturer's instructions. Na^+, K^+ -ATPase cell surface expression in C-peptide-treated MTALs was expressed as a percentage of cell surface expression in control MTALs. Quantification of Western blots was carried out using a Molecular Dynamics laser-scanning densitometer and the Imagequant software (Molecular Dynamics, Bucks., UK). Results were expressed as a percentage $\pm$ SE of the control optical density.

Adenylyl cyclase activity. Adenylyl cyclase activity was assessed on single microdissected MTALs by the conversion rate of α - ^{32}P -ATP to ^{32}P -cAMP under basal, C-peptide- and vasopressin-stimulated conditions [22]. Tubules were permeabilised by hypotonic shock and freeze-thawing and incubated at 30°C for 30 min with α - ^{32}P -ATP together with an ATP-regenerating system (phosphocreatine and creatine kinase). Thereafter, ^{32}P -cAMP was separated from other ^{32}P nucleotides by double column (Dowex and alumin) filtration procedure. The final yield of the whole separation procedure was evaluated in each sample by measuring recovered ^{3}H -cAMP added to each sample at the end of the incubation period. Results were expressed as fmol cAMP. $\text{mm}^{-1}30 \text{ min}^{-1} \pm \text{SE}$.

PKC translocation. For measurement of PKC translocation, outer medullary tubule suspensions were incubated in the absence of or with C-peptide after which 500 μl of ice-cold incubation solution was added. After a 1-min centrifugation at 400 g the pellet was resuspended in homogenisation buffer (in mmol/l: 250 sucrose, 100 Tris, 2 EDTA, 10 EGTA, 2 DTT, 1 PMSF, 1 AEBSF) and sonicated on ice. An aliquot of crude homogenate was taken for measurement of total PKC. The homogenate was then centrifuged at 135 000 g for 1 h at 4°C. The supernatant, containing the PKC cytosolic fraction was kept for analysis, and the pellet containing the membrane fraction was resuspended in 50 μl of homogenization buffer (see above). Protein concentration was evaluated by the bicinchoninic acid (BCA) assay (Pierce), and 50 to 100 μg protein were subjected to SDS-PAGE as previously described. Equal loading of different lanes was assessed by coomassie blue staining of a similar gel run in parallel.

After SDS-PAGE and electro transfer onto PVDF membranes, Western blotting was carried out using isoform-specific PKC antibodies. All PKC isoforms were detected on the same membrane to avoid loading differences. Quantification of

Western blots was done as described above and results were expressed as a percentage $\pm$ SE of the control optical density.

Na^+, K^+ -ATPase α -subunit phosphorylation. Phosphorylation of the catalytic α -subunit was assessed by Na^+, K^+ -ATPase immunoprecipitation after ^{32}P labelling of the α -subunit, as described elsewhere [21]. Outer medullary tubule suspensions were preincubated at 30°C for 2 h in oxygenated solution containing 37.10 Bq/ μl ^{32}P -orthophosphoric acid and then incubated with or without drugs and/or hormones. Tubules were then lysed in homogenisation buffer (see above) and protein concentration was calculated by the BCA assay. Identical amounts of protein (200 μg) were incubated overnight at 4°C with the anti- Na^+, K^+ -ATPase antibody together with saturating amounts of protein A-sepharose beads. Samples were then washed four times in ice-cold homogenisation buffer, once in 10 mmol/l Tris HCl pH 7.4, and resuspended in sample buffer. After separation by SDS-PAGE, proteins were electro transferred on PVDF membranes and autoradiography was carried out with hyperfilm-MP (Amersham-Pharmacia Biotech). Quantification of autoradiograms was done as described above for Western blots and results were expressed as a percentage $\pm$ SE of the control optical density.

Statistics. Comparison between two experimental groups was done by Student's *t* test for unpaired data. For multiple comparisons, one-way ANOVA with Scheffe's correction was used. A *p* value of less than 0.05 were considered statistically significant. All analyses were carried out using Statview software (Abacus Concepts, Berkeley, Calif., USA).

Results

To examine the role of C-peptide in the regulation of cation transport by MTAL, we first evaluated the concentration-dependence of its effect on Na^+, K^+ -ATPase activity under V_{max} conditions. Isolated MTALs were incubated with or without increasing concentrations of rat C-peptide ranging from 10^{-11} to 10^{-5} mol/l. This concentration range was chosen because it covers the physiological range of C-peptide circulating concentrations [23]. Figure 1A shows that after 15 min of incubation at 37°C, C-peptide dose-dependently stimulated Na^+, K^+ -ATPase activity in MTAL with a threshold close to 10^{-9} mol/l and a maximal effect obtained at 10^{-7} mol/l (as pmol Pimm $^{-1}\text{h}^{-1}$; control: 2182 ± 136 vs C-peptide: 3067 ± 576 ; $p < 0.01$). Time-dependence experiments using 10^{-7} mol/l C-peptide (Fig. 1B) showed a stimulation of Na^+, K^+ -ATPase activity after 5 min of incubation at 37°C that reached a plateau after 10 min (as pmol Pimm $^{-1}\text{h}^{-1}$; control: 2563 ± 76 vs C-peptide: 3487 ± 314 ; $p < 0.01$). Control experiments confirmed that scramble peptide did not alter Na^+, K^+ -ATPase activity (data not shown). These results indicate that physiological concentrations of C-peptide stimulate maximal Na^+, K^+ -ATPase activity in a saturable manner in rat MTAL.

The increase in maximal Na^+, K^+ -ATPase activity represents either a direct effect on maximal enzyme turnover or an increase in the number of active pump units. To discriminate between these two possibilities,

Fig. 1A, B. Concentration-dependence and time-course of the effect of C-peptide on Na^+, K^+ -ATPase activity. **A:** Isolated MTALs were incubated for 15 min at 37°C in the absence of (control, open square) or with increasing concentrations (10^{-11} to 10^{-5} mol/l, filled squares) of rat C-peptide. **B:** Isolated MTALs were incubated at 37°C for a total period of 60 min without (control, open square) or with addition of 10^{-7} mol/l C-peptide for 5 to 60 min (filled squares). Results are expressed as pmol $\text{ATPmm}^{-1}\text{h}^{-1}$ and are means $\pm$ SE from 3 to 10 independent experiments. *, $p < 0.05$

we measured the effect of C-peptide on Na^+, K^+ -ATPase cell surface expression using a biotinylation-streptavidin precipitation assay. Figure 2A shows that incubation of MTALs with 10^{-7} mol/l C-peptide for 15 min at 37°C did not alter the amount of cell surface Na^+, K^+ -ATPase α -subunit measured by Western blotting. Thus, C-peptide increases the turnover of Na^+, K^+ -ATPase in rat MTAL.

To evaluate the possibility of an additional effect of C-peptide on Na^+ affinity and/or on intracellular Na^+ concentration (secondary to an alteration in its apical entry) we measured the effect of C-peptide on the ouabain-sensitive ^{86}Rb uptake under rate limiting conditions and we calculated Na^+, K^+ -ATPase efficiency. As depicted in figure 2B, incubation of tubules with 10^{-7} mol/l C-peptide for 15 min stimulated ^{86}Rb uptake by about 30% (as pmol Rb. $\text{mm}^{-1} \cdot \text{min}^{-1}$; control: 16.7 ± 1.3 vs C-peptide: 21.8 ± 1.6 ; $p < 0.01$). For each experimental condition (control and 10^{-7} mol/l C-peptide for 15 min) Na^+, K^+ -ATPase efficiency was calculated as the ratio of the mean ouabain-sensitive ^{86}Rb uptake (Fig. 2B) and the mean Na^+, K^+ -ATPase activity (Fig. 2C) and assuming a stoichiometry of two Rb ions transported for each hydrolyzed ATP. We found that in intact MTAL cells, i.e. under intracellular Na^+ rate limiting conditions, Na^+, K^+ -ATPase functioned at 23.5% of its maximal capacity under basal

Fig. 2A–C. Effect of C-peptide on Na^+, K^+ -ATPase cell surface expression, ouabain-sensitive ^{86}Rb uptake and Na^+, K^+ -ATPase activity. Isolated MTALs were incubated for 15 min at 37°C in the absence of (control) or with 10^{-7} mol/l C-peptide. **A** A representative Western blot after Biotinylation procedure is shown (upper panel). Bars represent the densitometric quantification (means $\pm$ SE) from eight independent experiments. Results are expressed as a percentage of the control optical density. **B** Ouabain-sensitive ^{86}Rb uptake was measured under initial rate. Results are expressed as pmol $\text{Rbmm}^{-1}\text{min}^{-1}$ and are means $\pm$ SE from eight independent experiments. *, $p < 0.05$. **C** Na^+, K^+ -ATPase activity was measured as described in Fig. 1. Results are expressed as pmol $\text{ATPmm}^{-1}\text{h}^{-1}$ and are means $\pm$ SE from four independent experiments. *, $p < 0.05$

Fig. 3A, B. Effect of C-peptide on the amounts of membrane-bound PKC α , δ , ϵ and ζ isoforms. **A** Outer medullary tubule suspensions were sonicated and membrane fractions were obtained by ultracentrifugation. Equal amounts of protein (20 μg) from total homogenates (lanes 1, 3 and 5) and membrane fractions (lanes 2, 4 and 6) were separated by SDS-PAGE, transferred to PDVF membranes prior to Western blotting with anti- Na^+, K^+ -ATPase α -subunit and anti-aquaporin-2 antibodies. Results from three independent preparations are shown. **B** Outer medullary tubule suspensions were incubated without (control) or with 10^{-7} mol/l PMA or 10^{-7} mol/l C-peptide for 15 min at 37°C . PKC isoforms were detected by western blotting of membrane fractions (50 μg protein) with isoform-specific anti-PKC antibodies. A representative Western blot is shown (upper panel) and bars represent densitometric quantification (means $\pm$ SE) from seven independent experiments. Results are expressed as a percentage of the control optical density. *, $p < 0.01$.

conditions. The respective calculated efficiency for C-peptide-treated tubules was similar (22.5%), strongly suggesting that C-peptide did not alter Na^+, K^+ -ATPase Na^+ affinity and that its effect is independent of Na^+ availability.

The PKC- α , - δ , - ϵ and - ζ isoforms are expressed in rat renal outer medulla, but they do not share a common pattern of activation. Therefore, we analyzed the effects of C-peptide on the amounts of membrane-bound PKC isoforms using a cell fractionation procedure followed by Western blotting. The efficacy of the

Fig. 4A, B. Effect of PKC inhibition on C-peptide stimulation of Na^+, K^+ -ATPase activity and phosphorylation. **A** Isolated MTALs were incubated for 30 min at 37°C in the absence of or with 5.10^{-7} mol/l GF109203X (GFX) and 10^{-7} mol/l C-peptide was added (C-peptide) or not (control) for the last 15 min of incubation. Na^+, K^+ -ATPase activity was assessed as described in Fig. 1. Results are expressed as pmol $\text{ATPmm}^{-1}\text{h}^{-1}$ and are means $\pm$ SE from four independent experiments. *, $p < 0.05$. **B** Suspensions of outer medullary tubules were preincubated for 120 min at 30°C together with ^{32}P -orthophosphoric acid. Outer medullary tubules were then incubated for 30 min at 37°C in the absence of or with 5.10^{-7} mol/l GF109203X (GFX) and 10^{-7} mol/l C-peptide was added (C-peptide) or not (control) for the last 15 min of incubation. Phosphorylation of the Na^+, K^+ -ATPase α -subunit was revealed by autoradiography after immunoprecipitation. Bars represent the densitometric quantification (means $\pm$ SE) from seven independent experiments. Results are expressed as a percentage of the control optical density. *, $p < 0.01$.

membrane separation procedure is illustrated by the increase in Na^+, K^+ -ATPase α -subunit and aquaporin-2 immunoreactivities in membrane fractions with respect to total homogenates (Fig. 3A). Control experiments showed that incubation of outer medullary tu-

bule suspensions with $5 \cdot 10^{-7}$ mol/l phorbol myristic acid (PMA) for 10 min at 37°C increased the amounts of PKC- α in the membrane fraction, indicating that PKC translocation could be followed using this methodology. Figure 3B shows that incubation of outer medullary tubule suspensions for 15 min at 37°C with 10^{-7} mol/l C-peptide resulted in a specific increase in the amounts of the PKC- α isoform in the membrane fraction, while the amounts of PKC δ , ϵ and ζ isoforms remained unchanged. Thus, C-peptide specifically activates PKC- α in rat MTAL.

Since C-peptide stimulates Na^+, K^+ -ATPase and activates PKC- α , we assessed the effect of GF109203X, a specific PKC inhibitor, on C-peptide stimulation of Na^+, K^+ -ATPase activity in microdissected MTALs. Figure 4A shows that 10^{-7} mol/l GF109203X for 30 min at 37°C did not alter Na^+, K^+ -ATPase activity but completely abolished the stimulatory effect of 10^{-7} mol/l C-peptide added during the last 15 min of incubation, suggesting that stimulation of Na^+, K^+ -ATPase activity by C-peptide is PKC-dependent in rat MTAL.

Since in various cells PKC activation results in the phosphorylation of the catalytic Na^+, K^+ -ATPase α -subunit, we analyzed the effect of C-peptide on the phosphorylation amount of the Na^+, K^+ -ATPase α -subunit with or without GF109203X. As depicted by figure 4B, incubation of outer medullary tubule suspensions with 10^{-7} mol/l C-peptide for 15 min at 37°C increased phosphorylation of Na^+, K^+ -ATPase α -subunit to 160% of the control. The C-peptide-induced increase in the phosphorylation amount of the Na^+, K^+ -ATPase α -subunit was abolished by 10^{-7} mol/l GF109203X. These results strongly suggest that in MTAL, C-peptide stimulates Na^+, K^+ -ATPase activity by increasing the phosphorylation amounts of the Na^+, K^+ -ATPase catalytic α -subunit through PKC- α activation.

Discussion

Our study provides evidence that, within a physiological concentration range, C-peptide exerts a hormonal effect at the cellular level in rat MTAL. The results suggest that, in this segment of the nephron, C-peptide stimulates Na^+, K^+ -ATPase activity through the classical PKC- α signalling pathway. These results are consistent with a contribution of C-peptide to the multi-hormonal stimulation of Na^+, K^+ -ATPase activity during non-fasting periods.

C-peptide stimulation of Na^+, K^+ -ATPase activity in MTAL is in good agreement with previous studies that reported a concentration-dependent stimulation of Na^+, K^+ -ATPase activity by C-peptide in the rat proximal convoluted tubules (PCT) [6, 7] and human erythrocytes [27, 28]. However, in the PCT the threshold of C-peptide effect was about 1000 fold higher than in

MTAL, and thus far above the physiological range. In contrast, our results showed that in MTAL, effective concentrations of C-peptide are close to non-fasting plasmatic concentrations, suggesting that MTAL is more likely to be a physiological target of C-peptide than PCT. In addition, time-course experiments showed that C-peptide stimulated Na^+, K^+ -ATPase activity within 5 min. Altogether, these results strongly suggest that C-peptide participates in the short-term physiological control of Na^+, K^+ -ATPase activity, and thereby Na^+ handling, during post-prandial periods in MTAL. The effect of C-peptide could therefore counteract the higher MTAL Na^+ delivery secondary to the increase in GFR that occurs during the post-prandial period.

C-peptide stimulated Na^+, K^+ -ATPase activity measured in permeabilised tubules, i.e. in the absence of transmembrane ion gradients. This result indicates that Na^+, K^+ -ATPase was the primary target of C-peptide signalling and was not stimulated through an increase in Na^+ concentration brought about by an increase in apical Na^+ entry. Moreover, C-peptide did not alter the efficiency of Na^+, K^+ -ATPase, i.e. the proportion of active pump units under rate limiting intracellular Na^+ concentrations. In addition to intracellular Na^+ concentrations [29] Na^+, K^+ -ATPase efficiency is mainly assessed by Na^+, K^+ -ATPase Na^+ affinity [30] and to a lesser extent by K^+ conductance [31], and transmembrane voltage [32]. The identical efficiencies of Na^+, K^+ -ATPase under control or C-peptide-treated conditions would therefore argue against the participation of these regulatory factors in Na^+, K^+ -ATPase-mediated cation transport stimulation by C-peptide. This result is in contrast with the C-peptide-induced increase in Na^+ affinity of Na^+, K^+ -ATPase observed in rat proximal convoluted tubule [6] and points-out the cellular specificity of Na^+, K^+ -ATPase regulation [13]. On the other hand, the cell surface expression of the Na^+ -pump was not altered by C-peptide, therefore increased Na^+, K^+ -ATPase $V_{\max}$ values relies on an increased turnover rate. In agreement with this observation, cAMP synthesis, which results in stimulation of Na^+, K^+ -ATPase activity through an increase of Na^+, K^+ -ATPase cell surface expression, was not altered by C-peptide [21, 33].

Another aim of this study was to identify the intracellular signalling pathway that mediates the effect of C-peptide on Na^+, K^+ -ATPase activity. In MTAL, cation transport is stimulated in part by PKC-dependent mechanisms involving both basolateral and luminal transporters. Indeed, PKC activation by phorbol esters stimulated both apical $\text{Na}^+, \text{K}^+, 2\text{Cl}$ cotransporter [34] and basolateral Na^+, K^+ -ATPase [18]. Our results showed that C-peptide specifically increased the amounts of cell membrane associated PKC- α (an index of its activation [26]), while the amounts of membrane associated PKC- δ , - ϵ and - ζ isoforms remained unaltered. PKC- α activation is consistent with previ-

ous results showing that the effect of C-peptide on Na⁺,K⁺-ATPase activity is dependent on the pertussis toxin-sensitive G protein α -subunit and on an increase in intracellular Ca²⁺ concentration [6]. In addition, GF109203X abolished Na⁺,K⁺-ATPase activation and phosphorylation induced by C-peptide when used at concentrations that inhibit classical and novel PKCs but not atypical PKCs [35]. Together with its saturable effect, these findings strongly suggest that C-peptide acts through a G-protein coupled receptor linked to the classical PKC- α pathway in rat MTAL. Consistent with PKC- α translocation to the cell membrane, the results of this study show that C-peptide induced a PKC-dependent increase in Na⁺,K⁺-ATPase α -subunit phosphorylation amounts. Several investigators have shown that PKC- α can directly phosphorylate rat Na⁺,K⁺-ATPase α 1-subunit (the renal isoform) [36, 37] and that phorbol esters increase the phosphorylation amount of Na⁺,K⁺-ATPase α 1-subunit in a PKC-dependent manner in renal cells [18, 38, 39].

PKC-dependent phosphorylation of Na⁺,K⁺-ATPase α -subunit was associated with a PKC-dependent stimulation of Na⁺,K⁺-ATPase activity, suggesting that both processes are linked together. However, previous studies have shown that PKC phosphorylation of the Na⁺,K⁺-ATPase α 1-subunit is associated with either inhibition or stimulation of Na⁺,K⁺-ATPase activity [18, 39, 40, 41, 42]. These differential effects rely on metabolic factors [43], intracellular calcium concentrations [44], PKC isoforms [38], as well as cell specific factors [45, 46]. Taken together, all these results suggest that PKC phosphorylation of the α 1-subunit does not directly alter Na⁺,K⁺-ATPase intrinsic properties but rather could recruit cell-specific regulatory factors that determine the functional effect of Na⁺,K⁺-ATPase phosphorylation.

In summary, our results indicate that in MTAL C-peptide exerts a hormonal effect at a cellular level through PKC- α -dependent Na⁺,K⁺-ATPase phosphorylation. From a physiological point of view, the active concentrations of C-peptide on Na⁺,K⁺-ATPase are within a post-prandial plasma concentration range. This result is consistent with a contribution of C-peptide to Na⁺,K⁺-ATPase stimulation and thereby to renal Na⁺ handling during non-fasting periods.

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